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Kinetic Concepts for Measuring Microbial Rate Constants: Effects of Nutrients on Rate Constants

D. F. PARIS* AND J. E. ROGERS

Environmental Research Laboratory, U.S. Environmental Protection Agency, Athens, Georgia 30613

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We investigated the effect of preincubation of environmental waters amended with inorganic nutrients (nitrogen, phosphorus, and traces of iron and magnesium) on the kinetics of the microbial transformation of phenol, propanil, propyl ester of (2,4-dichlorophenoxy)acetic acid, methyl parathion, Ronnel, and methoxychlor in pond and river waters. No effect on the second-order rate constants for these compounds was observed, although there was an increase in the bacterial populations and the pseudo-first-order rate constants. The use of nutrient-amended waters could be a useful tool for estimating second-order rate constants for an expanded number of compounds. This technique would provide a larger data base for predicting the behavior of xenobiotic compounds in the environment by using currently available mathematical models.

With the ever-increasing sophistication of computer programs to model the degradation of xenobiotic compounds released into environmental waters, it is no longer sufficient simply to know that a compound is or is not biodegradable. To adequately estimate ecosystem effects and persistence of these chemicals, we now need accurate measures of, for example, the rate of xenobiotic transformation (degradation, mineralization, etc.), the formation rate of potentially toxic intermediates, and the effects of environmental conditions on the transformation processes.

Several kinetic approaches for describing the transformation of xenobiotic compounds by suspended microorganisms in aquatic environments are currently being evaluated (2, 6, 10, 12, 16, 17). Prominent among these approaches is the use of second-order kinetics. The second-order rate expression depends on the concentration of the xenobiotic compounds and the active bacterial population.

$$dS/dt = k_bBS \quad (1)$$

This rate expression, in which S is the xenobiotic concentration, B is the bacterial concentration, and k_b is the second-order rate constant, is a reduced form of the Monod expression (equation 2), in which the concentration of the xenobiotic compound is much less than K_s.

$$dS/dt = \mu_mBS/Y(K_s + S) \quad (2)$$

For the limiting condition, the second-order rate constant (k_b) is equated to μ_m/YK_s (μ_m is maximum growth rate, Y is the yield factor, and K_s is the half-saturation constant). Equivalence of μ_m/YK_s and k_b has been demonstrated experimentally for systems in which the substrate was the sole carbon source and second-order kinetics were observed (9).

In general, second-order rate constants are determined by forcing the reaction to proceed in a pseudo-first-order fashion by maintaining one substrate (the fixed substrate) in great excess over the other. In this way the reaction appears to be first-order with respect to the limiting substrate, and a pseudo-first-order constant can be determined from a semilogarithmic plot of residual limiting-substrate concentration versus time. The second-order constant is then obtained by dividing the first-order constant by the concentration of fixed substrate. If the second-order constant does not change when the fixed-substrate concentration is varied, the reaction is said to be second order. In determining the second-order rate constant for transformations of xenobiotic compounds by suspended aquatic microorganisms, the bacterial population determined by plate counts is considered the fixed substrate, and the xenobiotic concentration is considered the limiting substrate.

The reliability of second-order rate constants for assessing microbial degradation kinetics in natural waters has been demonstrated for the butoxyethyl ester of 2,4-dichlorophenoxyacetic acid (10), malathion (10), chlorpropham (10), a series of substituted phenols (11), and other compounds. Although the rate constants varied naturally between compounds, the rate constants for a given compound were remarkably similar (less than ±75%) over a wide range (10 to 40) of aquatic samples having bacterial populations ranging from 4.8×10^5 to 9.8×10^8 CFU/liter. Also, the pseudo-first-order transformation constants for the butoxyethyl ester of 2,4-dichlorophenoxyacetic acid were proportional to the bacterial concentration, and the second-order rate constants were not significantly different at the 95% confidence level when bacterial population sizes were experimentally manipulated by resuspending microorganisms (concentrated by filtration) to population sizes ranging over 2 orders of magnitude.

For xenobiotic compounds that are slowly biodegraded, the determination of second-order rate constants is generally limited by the concentration of natural bacterial populations in test waters. Fortunately, however, enhancement of the natural bacterial population by preincubation with added nutrients permits the determination of second-order constants for some compounds. For example, second-order rate constants determined for chlorpropham with natural and nutrient-enhanced populations were equivalent (10). The usefulness of enhanced microorganisms is further substantiated in the present study in which we show that the addition of inorganic nutrients to increase natural populations does not alter second-order rate constants determined for phenol, propanil, propyl ester of 2,4-dichlorophenoxyacetic acid (DPE), methyl parathion (MP), Ronnel, and methoxychlor.

MATERIALS AND METHODS

Experimental design. Second-order rate constants for the biotransformation of phenol, propanil, DPE, MP, Ronnel,

* Corresponding author.

TABLE 1. Characteristics of environmental waters

Site	TOC[a] (mg/liter)	NH_3 (mg/liter)	NO_3-NO_2N (mg/liter)	Total P (mg/liter)
Hickory Hills pond	7.7	0.06	0.08	0.02
Memorial Park pond	8.2	0.14	0.40	0.02
Oconee River	7.8	0.70	2.00	0.26
Water Works pond	6.8	0.05	0.11	0.02

[a] TOC, Total organic carbon.

and methoxychlor were determined for nutrient-amended and unamended environmental waters. For the latter two compounds, bacterial populations in water samples were concentrated 10-fold by filtering 10 liters of water through washed membrane filters (0.22-μm pore diameter; Millipore Corp., Bedford, Mass.), suspending the filters in 1 liter of the environmental water, and stirring the water for approximately 1 h before removing the filters. The enriched water was used in the experiments in the same way as the unamended water samples.

Environmental water samples from four sites (Table 1) within 10 km of Athens, Ga., were collected in polyethylene bottles and transported to the laboratory, where each sample was divided into two portions. To one portion was added 0.5 g of NH_4Cl, 0.5 g of $(NH_4)_2SO_4$, 0.05 g of Na_2HPO_4, 0.05 g of KH_2PO_4, 0.001 g of $MgSO_4$, and 0.001 g of $FeCl_3$ per liter from sterile aqueous stock solutions. Both portions were incubated 12 h overnight at 22°C to yield an untreated and an enhanced bacterial suspension. This procedure enhanced the bacterial population 10 to 100 times in the treated sample. After incubation, 200-ml samples of each portion were dispensed into a series of 500-ml Erlenmeyer flasks. Autoclaved and filter-sterilized water samples served as controls for abiotic processes.

Individual experiments were initiated by the addition of a 10- to 20-μl acetone solution of each compound. Experiments with enhanced waters were conducted at two compound concentrations an order of magnitude apart, whereas experiments with unamended and sterile waters were conducted with a single substrate concentration. All compound concentrations were 1 mg or less per liter of water. Control experiments without added xenobiotic compounds served to detect changes in bacterial population not due to the presence of the xenobiotic compound. All flasks were incubated at 22°C in a temperature-controlled shaker. All experimental flasks were subsampled at prescribed intervals to measure concentrations of residual xenobiotic compounds. Bacterial levels were enumerated at prescribed times by pour plate counts. Biological activity was terminated by the addition of extraction solvent or 50 μl of a 37% Formalin solution.

Concentrations of residual xenobiotic compounds were monitored by high-pressure liquid chromatography of centrifuged samples or a combination of solvent extraction and gas-liquid chromatography analysis. At the end of each experiment, organic extracts of the flask contents were analyzed by thin-layer chromatography for products.

Extractions and chromatography. Residual levels of methoxychlor, MP, DPE, propanil, and Ronnel were measured by a combination of solvent extraction (isooctane) and gas-liquid chromatography. Gas-liquid chromatographic analyses were conducted with a Tracor MT-222 gas-liquid chomatograph equipped with a ^{63}Ni high-temperature electron capture detector and a 1.5-m-long column (4-mm inner diameter) packed with 3% SE-30 on 80/100-mesh Gas-Chrom Q (Alltech Associates, Inc., Applied Science Div., State College, Pa.). Nitrogen was the carrier gas at a flow of 60 ml/min; operating temperatures for the inlet port and detector were 250 and 325°C, respectively.

Concentrations of residual phenol were measured by high-pressure liquid chromatography. Reactions were stopped by the addition of Formalin, and the samples were centrifuged to remove microorganisms and suspended particulate matter. Supernatant solutions were analyzed by direct injection into a 970 A Tracor liquid chromatograph equipped with a Whatman octadecylsilane-2 column and a UV light detector (272 nm). The mobile-phase solution ratio was 35 parts acetonitrile/64.8 parts water/0.2 part acetic acid. The flow rate was 2 ml/min, and the pressure was 1,400 lb/in^2.

The primary products of xenobiotic biotransformation (Table 2) were identified by a combination of solvent extraction and thin-layer chromatography. Water samples from experiments with MP, DPE, Ronnel, and phenol were acidified with 6 N hydrochloric acid before extraction. MP, methoxychlor, propanil, and Ronnel were extracted with methylene chloride. Phenol and DPE were extracted with ethyl acetate and diethyl ether, respectively. Products in the solvent extracts were cochromatographed on thin-layer chromatography plates coated with silica gel containing a fluorescent indicator. Developing solvents and R_f values are shown in Table 2.

Enumeration of bacteria. Bacterial populations were enumerated by standard pour plate techniques with 5 to 7 ml of tryptone glucose extract agar (Difco Laboratories, Detroit, Mich.) per plate. Pour plates were incubated for 48 h at 22°C before colonies were counted.

Individual colonies isolated from enumeration plates were inoculated into 1:10 nutrient broth containing the test compound at the same concentration used in the corresponding biotransformation study. Cultures showing a 30% or greater loss of the test compound were considered active transformer colonies (8).

Transformation kinetics. Pseudo-first-order rate constants (k_1) were obtained from a least-squares regression of the log plot of substrate concentration versus time. Second-order rate constants (k_b) were obtained by dividing k_1 by the

TABLE 2. Thin-layer chromatography solvents and R_f values for xenobiotic compounds and their products

Developing solvent (relative vol)	Parent compound (R_f)	Product[a] (R_f)
Ethyl ether (1) Pet ether (9)	Methoxychlor (0.25)	Methoxychlor DDE (0.20)
Benzene (45) Methanol (10) Acetic acid (1)	Phenol (0.40)	Catechol (0.45)
Benzene (75) Methanol (25)	MP (0.78)	*p*-Nitrophenol (0.32)
Benzene (75) Methanol (25)	Ronnel (0.75)	2,4,5-Trichlorophenol (0.47)
Benzene (75) Methanol (25)	Propanil (0.45)	3,4-Dichloroaniline (0.64)
Benzene (75) Methanol (25)	2,4-DPE (0.83)	2,4-D (0.40)

[a] Methoxychlor DDE, 1,1 dichloro-2,2-bis(*p*-methoxyphenyl)ethylene; 2,4-D, 2,4-dichlorophenoxyacetic acid.

TABLE 3. Bacterial concentrations (B) and pseudo-first-order (k_1) and second-order (k_b) rate constants for sites used in methoxychlor transformation studies[a]

Site and kind of sample	k_1 (h^{-1})	B (organisms per liter)	$10^{-13}\ k_b$ (liters · organisms^{-1} · h^{-1})
Oconee River			
Amended	0.006–0.009	$(2.0–4.6) \times 10^{10}$	3.9 ± 3.0
Unamended	0.001–0.004	$(2.0–8.5) \times 10^{9}$	4.2 ± 3.4
Hickory Hills pond			
Amended	0.008–0.012	$(4.1–5.7) \times 10^{10}$	2.0 ± 1.2
Unamended	0.002–0.003	$(7.1–8.0) \times 10^{9}$	3.4 ± 2.5
Memorial Park pond			
Amended	0.007–0.011	$(2.3–6.1) \times 10^{10}$	1.7 ± 1.2
Unamended	0.001–0.006	$(2.0–8.2) \times 10^{9}$	1.2 ± 1.0
Water Works pond			
Amended	0.006–0.008	$(1.7–4.1) \times 10^{10}$	3.7 ± 2.7
Unamended	0.002–0.004	$(5.0–8.1) \times 10^{9}$	2.1 ± 1.4

[a] These results were compiled from eight determinations for amended waters and four determinations for unamended waters.

bacterial concentration (B). Because bacterial concentrations did not change significantly during the experiments and the changes were random and not indicative of bacterial growth, the concentrations were averaged, and this value was used for B.

Materials. Phenol was purchased from Aldrich Chemical Co., Milwaukee, Wis.; DPE was prepared by acid-catalyzed reaction of the commercial acid (Aldrich) with propyl alcohol; the ester was purified by fractional distillation. MP was purchased from Supelco, Inc., Bellefonte, Pa.; propanil, methoxychlor, and Ronnel were obtained from the Pesticides and Industrial Chemicals Repository, Research Triangle Park, N.C.

RESULTS

We have examined the effects of added inorganic nutrients on the biotransformation of DPE, phenol, propanil, MP, Ronnel, and methoxychlor. These compounds are representative of five biologically catalyzed processes that include ester hydrolysis of DPE, amide hydrolysis of propanil, phosphoester hydrolysis of MP and Ronnel, ring hydroxylation of phenol, and dechlorination of methoxychlor. The immediate transformation products for the six compounds appeared to be the major products when solvent extracts of waters from experiments with all six compounds (Table 2) were analyzed by thin-layer chromatography.

Preincubation with inorganic nutrients increased both the bacterial populations and the pseudo-first-order rate constant for biotransformation, whereas the second-order rate constants were essentially unaffected. Examples of the results for all three parameters are shown in Tables 3 and 4 for the compounds methoxychlor and phenol. Although the effects of added nutrients were not as pronounced for methoxychlor (Table 3) as they were for phenol (Table 4), significant increases in both the bacterial population and the pseudo-first-order rate constant were observed with the two compounds. In contrast, the second-order rate constants did not vary significantly between amended and unamended waters. A summary of the average second-order rate constants determined for amended and unamended waters for all six compounds is shown in Table 5.

For methoxychlor, MP, and Ronnel, a lag period which we define as time before biotransformation could be detected as a first-order rate process was observed. Preincubation of the water samples in the presence of nutrients reduced this period by 30 to 40%. An example of these results is shown in Fig. 1.

Examination of individual colonies isolated from enumer-

TABLE 4. Bacterial concentrations (B) and pseudo-first-order (k_1) and second-order (k_b) rate constants for sites used in phenol transformation studies[a]

Site and kind of sample	k_1 (h_{-1})	[B] (organisms per liter)	$10^{-10}\ k_b$ (liters-organisms^{-1} · h^{-1})
Oconee River			
Amended	0.120–0.690	$(4.0–8.0) \times 10^{8}$	2.1 ± 1.1
Unamended	0.005–0.009	$(1.0–5.1) \times 10^{7}$	2.0 ± 1.0
Hickory Hills pond			
Amended	0.034–0.083	$(1.1–2.8) \times 10^{8}$	3.1 ± 1.7
Unamended	0.005–0.008	$(0.5–4.9) \times 10^{7}$	2.1 ± 0.8
Memorial Park pond			
Amended	0.028–0.064	$(3.9–6.7) \times 10^{8}$	2.3 ± 1.3
Unamended	0.006–0.009	$(0.4–8.0) \times 10^{7}$	4.4 ± 2.2
Water Works pond			
Amended	0.290–0.450	$(1.1–1.9) \times 10^{9}$	3.2 ± 2.4
Unamended	0.003–0.006	$(2.0–2.4) \times 10^{7}$	2.5 ± 1.9

[a] These results were compiled from eight determinations for amended waters and four determinations for unamended waters.

TABLE 5. Microbial transformation rate constants (k_A)[a] for six compounds in amended and unamended waters

Site and kind of sample	Liters · organisms^{-1} · h^{-1}					
	10^{-9} DPE	10^{-10} Phenol	10^{-11} Propanil	10^{-11} Methyl parathion	10^{-11} Ronnel	10^{-13} Methoxychlor
Oconee River						
Amended	3.7 ± 2.8	2.1 ± 1.1	3.8 ± 1.4	2.0 ± 1.3	3.1 ± 1.0	3.9 ± 3.0
Unamended	1.9 ± 0.9	2.0 ± 1.0	2.7 ± 1.5	1.6 ± 0.9	2.2 ± 1.6	4.2 ± 3.4
Hickory Hills pond						
Amended	2.7 ± 1.2	3.1 ± 1.7	1.8 ± 0.8	1.2 ± 0.8	2.0 ± 1.5	2.0 ± 1.2
Unamended	2.9 ± 2.3	2.1 ± 0.8	1.5 ± 0.7	4.5 ± 3.6	0.9 ± 0.7	3.4 ± 2.5
Memorial Park pond						
Amended	2.1 ± 1.0	3.1 ± 1.7	1.8 ± 0.8	1.2 ± 0.8	2.0 ± 1.5	2.0 ± 1.2
Unamended	1.9 ± 0.8	4.4 ± 2.0	3.0 ± 1.1	2.5 ± 1.5	2.3 ± 1.0	1.2 ± 1.0
Water Works pond						
Amended	2.6 ± 1.5	3.1 ± 2.4	4.5 ± 3.7	2.5 ± 1.4	2.9 ± 1.9	3.7 ± 2.7
Unamended	3.2 ± 1.9	2.5 ± 1.9	2.9 ± 2.1	1.4 ± 1.2	2.1 ± 1.8	2.1 ± 1.4
Mean						
Amended	2.8 ± 0.7	2.7 ± 0.5	3.7 ± 1.3	2.1 ± 0.6	2.9 ± 0.6	2.8 ± 1.1
Unamended	2.5 ± 0.7	2.8 ± 1.1	2.5 ± 0.7	2.5 ± 1.4	1.9 ± 0.7	2.7 ± 1.3

[a] Mean and standard deviation of eight determinations for amended waters and four determinations for unamended waters.

ation plates prepared during the course of two experiments for each of the four different waters samples indicated that most colonies were capable of transforming the particular xenobiotic being investigated. In each instance, 25 colonies were examined for a total for each compound of 100 colonies per treatment, nutrient amended and unamended. No significant difference in the percentage of transforming organisms was observed between nutrient-amended or unamended waters. All bacteria examined transformed phenol, propanil, DPE, and Ronnel, and 85 to 92% of the tested bacteria were positive transformers of MP and methoxychlor.

DISCUSSION

In the present study, we examined the microbial transformation of six xenobiotic compounds in unamended and inorganic-amended samples of environmental waters. We found that although the preincubation of environmental waters with inorganic nutrients in batch culture led to an increase in the rate of transformation of a series of xenobiotic compounds when compared with untreated waters, no differences were observed for second-order rate constants derived from these experiments. The invariance of the second-order rate constants apparently resulted from an increase in transformation rate because of a corresponding proportionate increase in the number of transformer microorganisms in amended samples. This combination of results could occur only if, as we found, the percentage of transformers was the same in both amended and unamended waters.

Numerous experimental approaches (3–5, 7, 13, 15, 16, 19) have been used to investigate the effects of organic and inorganic nutrients on the transformation or mineralization of xenobiotic compounds in natural waters. Schmidt and Alexander (15) found that secondary organic substrates played an important role in controlling the rate and extent of the biodegradation of organic compounds at low concentrations. For example, mineralization of 2.0 μg of phenol per liter by *P. acidoronans* was delayed 16 h by the addition of 17 μg of acetate per liter, and the delay was lengthened further by increasing the concentration of acetate. Mineralization of both substrates occurred simultaneously when the initial concentration of acetate was 13 μg/liter or less. Additionally, when *Pseudomonas* sp. strain ANL was grown in a salts solution supplemented with 300 μg each of glucose and aniline, the sugar was mineralized first and aniline was mineralized only after much of the glucose carbon was converted to CO_2. Working with natural microbial communities from a mesotrophic reservoir, Shimp and Pfaender (16) found that adaption to increasing concentrations of amino acids, carbohydrates, or fatty acids enhanced the ability of the microbial community to degrade a series of phenols. Unfortunately, the mechanism responsible for the enhanced

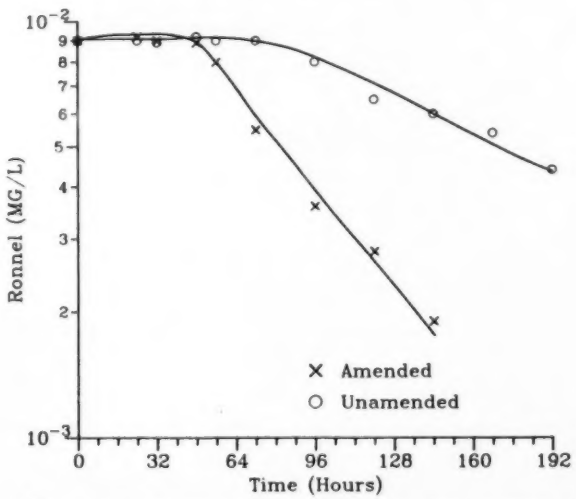

FIG. 1. Plots of Ronnel concentration versus time in amended and unamended waters. Bacterial concentrations are 1.1×10^9 organisms per liter in amended and 1.8×10^8 organisms per liter in unamended waters.

degradation was not clearly identified; the authors, however, suggested that the enhanced degradation resulted from a general stimulation of microbial metabolism. Rubin and Alexander (13) observed that rates of phenol mineralization in samples of three lakes were directly proportional to phenol concentration, although the rate at any one concentration was related to the trophic level of the water. In a related study, Rubin et al. (14) found the extent of mineralization of phenylacetate and *p*-nitrophenol to be greater in a eutrophic lake than in a mesotrophic lake. The effects of inorganic nutrients have been examined by workers in several laboratories (3, 4, 7, 19). Dibble and Bartha (3) reported that mineral nutrient ratios of 60:1 (C/N) and 800:1 (C/P) resulted in the greatest amount of degradation of oil sludge on soil. Wang et al. (19) observed that isopropyl *N*-phenylcarbamate (400 pg/ml) mineralization in lake water was enhanced by the addition of inorganic nutrients. Working with pure cultures, Lewis et al. (7) found that enhanced degradation of the butoxyethyl ester of 2,4-dichlorophenoxyacetic acid by added nutrients resulted from an increase in total biomass while the per-cell transformation rate remained constant. Similar results were reported by Paris et al. (10) for the transformation of chloropropham to 3-chloroaniline by microorganisms in environmental waters. Our current work with natural waters in which we have examined the effect of preincubation with inorganic nutrients on the second-order rate constants for the transformation of a series of compounds also supports the conclusion of Lewis et al. (7).

Several studies (7, 9, 18) have shown that the biotransformation of a number of xenobiotic compounds can be described by second-order kinetics and that a reasonably consistent second-order rate can be measured over a wide range of environmental waters. Implicit in these studies has been the assumption that the number of transformer microorganisms in environmental water samples is a relatively constant proportion of the measured microbial community used to calculate the second-order rate constant. The consistent proportion of transformer organisms observed here for all six compounds supports this assumption. Because the second-order rate constants derived from amended and unamended samples were invariant, the average transformation rate per organism must not have been affected. Apparently preincubation nonselectively enhanced transformation activity by increasing the bacterial population without markedly altering species diversity.

Increasing transformation activity without changing the second-order rate constant has certain practical implications. Most currently operational exposure models (1) use a second-order rate expression to describe the biotransformation of xenobiotic compounds in environmental waters. In such models, disappearance rates for compounds introduced to environmental waters are calculated from the concentrations of the indigenous bacteria and the xenobiotic compound and from an estimate of the second-order rate constant. The utility of these models is limited, therefore, by the availability of second-order rate constants. Because of the inability to maintain a stable environmental microbial population in the laboratory for much longer than a week, the estimation of second-order rate constants has been restricted to those compounds having rate constant values greater than 10^{-12}. In this study, preincubation led to a 10- to 100-fold increase in bacterial population without affecting the second-order rate constant. This procedure could extend the number of compounds for which second-order rate constants could be determined to include compounds with rate constants as low as 10^{-14}.

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